REMARKS

Upon entry of the instant amendment claims 24, 28, 30, 35 to 46, 49 and 50 are pending, of which claims 28 and 36 where withdrawn from consideration by the Examiner. Thus, no new matter has been added.

The Office Action dated February 7, 2008 has been carefully reviewed and the following reply is made in response thereto. In view of the following remarks, Applicants respectfully request reconsideration and reexamination of this application and the timely allowance of the pending claims.

The rejection of claims 24, 30-31, 35, 39-46 and 48-49 as being anticipated under 35 U.S.C. § 102 (b) by Konig *et al.* (WO 96/25435)

The Examiner has rejected claims 24, 30, 31, 35, 39-46, 48 and 49 under 35 U.S.C. § 102 (b) as being anticipated by Konig *et al.* (WO 96/25435) (Konig). Specifically, the Examiner asserts that the antibodies inherently opsonize and further asserts that Applicants have not provided any facts that the antibody of Konig fails to work *via* opsonization.

Applicants respectfully submit that the Examiner has not met his burden of establishing a *prima facie* case of anticipation. Applicants respectfully submit that a claim is anticipated only if each and every element at set forth in the claim is found in the prior art reference. *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631 (Fed. Cir. 1987). Applicants assert that Konig does not teach each and every limitation of the claimed invention.

As stated by Konig, antibody 369.2B binds to the C-terminal end of the Aβ peptide (see Konig p. 6, lines 13 to 21). Applicants herein submit the publication of Bard *et al.* (2003) PNAS, 100, 2023 to 2028 (Bard) (Exhibit A), that states that "[w]e have shown previously that not all antibodies against Aβ can trigger plaque clearance *in vivo*." (Bard at p. 2024, first column, first sentence of *Results and Discussion*). Bard further states that C-terminal antibodies were inactive and were not effective in reducing plaques *in vivo*. (*See* Bard at p. 2024, first column, last sentence of of first paragraph of *Results and Discussion*). Since the 369.2B antibody is a member of the Aβ C-terminal binding class of antibodies, there is strong evidence that 369.2B will not be effective in reducing plaques *in vivo*.

In addition, Applicants herein provide a Declaration under 37 C.F.R. § 1.132 signed by Mr. Stephen Wood providing data collected by Amgen, Inc., the licensee of the instant application, ("Wood Declaration"). Specifically, binding assays against Aβ42 with mAb 369.2B show that this antibody has weak binding when compared to control antibody, 2.1 chimera. (Wood Declaration at ¶ 5). In addition, immunohistochemistry performed on unfixed brain tissue of fresh-frozen sections from an 18 month-old Tg2576 mouse show little binding of mAb 369.2B to plaques in the brain tissue when compared to 2.1 chimera. (Wood Declaration at ¶ 6). As stated, these data show that the antibody disclosed in Konig, 369.2B, has weak binding and that the low-level binding exhibited by mAb 369.2b is not sufficient to opsonize amyloid fibrils and would not be predicted to induce removal of amyloid deposits *in vivo*.

The Wood Declaration also presents data that show that 369.2B does not direct phagocytosis in an *ex vivo* assay. It has been shown previously that the phagocytosis assay described in the Wood Declaration is the best available predictor of *in vivo* antibody efficacy (reduction of plaque burden) in mouse models of Alzheimer's Disease.[1] Specifically, unfixed brain sections from a 19 month-old Tg2576 mouse and microglial IC21 cells (a phagocytic cell-line) were incubated with several C-terminal Aβ binders, including mAb 369.2b. After 24-hours of incubation, the tissue samples were fixed, stained and scored for phagocytic events. Data in the Wood Declaration show that mAb 369.2b does not induce phagocytotic activity. (Wood Declaration at ¶ 8). Thus, data in the Wood Declaration show that the antibody disclosed in Konig, 369.2B, has weak binding and does not induce *in vitro* phagocytotic activity.

In summary, Bard states that C-terminal antibodies (*e.g.* mAb 369.2B) are inactive and are not effective in reducing Aβ plaques *in vivo* and the Wood Declaration presents evidence that 369.2B exhibits only weak binding to amyloid fibrils and 369.2b does not have *in vitro* phagocytosis activity. Thus, this is evidence that mAb 369.2b will not opsonize and remove plaques *in vivo*, as claimed.

Applicants also submit that Konig does not disclose human antibodies as require by claim 31. Although the Examiner states that Konig does, because, "[a]s first stated at p. 5 of the office action dated 30 September 2003, 'the antibodies include human…see in particular p. 22-24.'" Applicants submit that on page 22-24 of Konig there is no mention of human antibodies. In fact,

there is no mention of using human antibodies in the entire specification. Thus, this reference cannot anticipate claim 31 for this added reason.

Thus, in view of Bard and the Wood Declaration, Applicants assert Konig does not anticipate rejected claims and the rejection under 35 U.S.C. § 102(b), in view of Konig, must be withdrawn.

The rejection of claims 24, 30-35 and 37-49 as being anticipated under 35 U.S.C. § 102(b) by Becker *et al.* (EP 613,007)

The Examiner has rejected claims 24, 30-35 and 37-49 under 35 U.S.C. 102(b) as being anticipated by Becker *et al*, (EP613007) (Becker). Specifically, the Examiner states "administration of the antibodies for therapeutic purposes would nonetheless inherently result in the opsonization of amyloid deposits as currently claimed." (See, February 7, 2008 Office Action, p. 8).

Applicants submit that the Examiner has not met his burden of establishing a *prima facie* case of anticipation. Applicants respectfully to point out that "[a] claimed invention cannot be anticipated by a prior art reference if the allegedly anticipatory disclosures cited as prior art are not enabled." *Amgen v. Hoechst Marion Roussel, Inc.* 314 F.3d 1313, 1354 (Fed. Cir. 2003).

Applicants assert that Becker does not anticipate the claims because Becker does not enable a person of skill in the art to use the invention. Specifically, Becker fails to demonstrate an antibody to administer to a patient to remove amyloid deposits, as required by the claims. The Examiner asserts that because Becker states that the antibodies disclosed can be used therapeutically, "[o]ne of skill in the art would immediately recognize that such would mean administration of the antibody in an amount sufficient to elicit an effective (i.e. therapeutic) response." (February 7, 2008 Office Action, page 8). However, a person of skill in the art after reviewing Becker would require performing an undue amount of experimentation to create and select an antibody to administer to a patient to remove amyloid deposits *in vivo*. There is no guidance in Becker for one of skill in the art to determine whether an antibody would opsonize the amyloid deposits, as required by the presently claimed invention. Although the Examiner asserts that any antibody would inherently opsonize a target, Applicants submit that this is not

[1] Bard, F. *et al.* (2003) PNAS 100(4), 2023-8 (Exhibit A).

true. Applicants have submitted evidence showing that not all antibodies against Aβ opsonize β-amyloid plaques (see Wood Declaration and Bard *supra*). Thus, Becker does not enable a person of skill in the art to make an antibody and test for opsonization of an amyloid plaque as required by the claims.

In addition, Applicants respectfully submit that a claim is anticipated only if each and every element at set forth in the claim is found in the prior art reference. *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631 (Fed. Cir. 1987). Applicants assert that Becker does not teach each and every limitation of the claimed invention. The Examiner has alleged that "administration of the antibodies for therapeutic purposes would nonetheless inherently result in the opsonization of amyloid deposits as currently claimed." (See, February 7, 2008 Office Action, p. 8). Applicants disagree with this statement. Applicants respectfully submit that the fact that a certain result or characteristic may occur or be present in the prior art is not sufficient to establish the inherency of that result or characteristic. *In re Rijckaert*, 9 F.3d 1531, 1534 (Fed. Cir. 1993). In addition in *In re Oelrich*, 666 F.2d 578 (C.C.P.A.1981), the court states that inherency "may not be established by probabilities or possibilities. The mere fact that a certain thing may result from a given set of circumstances is not sufficient." *Id* at 581. Also, in *In re Robertson*, 169 F.3d 743 (Fed. Cir. 1999), the court stated that the mere fact that a certain thing may result from a given set of circumstances is not sufficient to establish inherency. *Id* at 745.

As discussed above, Applicants have submitted Bard (Exhibit A) which states "[w]e have shown previously that not all antibodies against Aβ can trigger plaque clearance *in vivo*." Bard at p. 2024. Bard further states that C-terminal antibodies were inactive and were not effective in reducing plaque *in vivo*. (See Bard at p. 2024, first column, first paragraph of *Results and Discussion*). Thus, contrary to the Examiner's statements, not all antibodies "inherently result in the opsonization of amyloid deposits as currently claimed." Thus, since anticipation "may not be established by probabilities or possibilities," Becker cannot anticipate the instant claims.

The Examiner further asserts that "...there are many other examples of antibodies that are effective (e.g. see Konig et al. reference)." (February 7, 2008 Office Action, p. 8). As stated in Bard, not all antibodies can trigger plaque clearance *in vivo*. Further, Applicants have submitted data in the Wood Declaration that shows that the antibody disclosed in Konig has weak binding

and does not have *in vitro* phagocytosis activity. Applicants submit that these experiments are predicative of how an antibody will perform *in vivo* and there is strong evidence that 369.2B will not opsonize an amyloid fibril and induce removal of amyloid deposits.

Thus, in view of the above arguments, Applicants assert that Becker does not anticipate the instant claims because Becker do not enable a person of skill in the art to determine an antibody to be administered to a patient to remove amyloid deposits *in vivo*. In addition, Becker does not inherently anticipate the claims because, as shown in the Wood Declaration and in Bard, not all antibodies opsonize. Thus, Applicants request that the rejection under 35 U.S.C. 102 (b), in view of Becker, be reconsidered and withdrawn.

The rejection of claim a obvious under 35 U.S.C. § 103 (a) in view of Konig *et al.* (WO 96/25435)

The Examiner has rejected claim 50 under 35 U.S.C. § 103 (a) as being unpatentable in view of Konig. Specifically, the Examiner alleges that Konig (at p. 13, lines 16-20 and at p.25, lines 13 to 17) teaches methods of preventing aggregation of β-amyloid and disrupting aggregation in Alzheimer's disease and that the antibody is useful for monitoring the level of β-amyloid peptide in the treatment of Alzheimer's disease. (February 7, 2008 Office Action, pp. 10-11). Thus, in view of these teachings the Examiner alleges that it would have been obvious to a person of skill in the art to provide maintenance doses and that because the reference teaches prevention and monitoring the progression of the disease, it would have been obvious to assess the patient's status and provide maintenance doses.

Applicants traverse this rejection and assert that the Examiner has not met his burden of establishing a *prima facie* case of obviousness. "To establish *prima facie* case of obviousness of a claimed invention, all claim limitation must be taught or suggested by the prior art" (MPEP 2143.03).

Claim 50 is dependent on claim 24, which requires that an antibody administered to a patient remove amyloid deposits *via* opsonization. As stated above, Konig does not disclose an antibody that will remove amyloid deposits *in vivo*. See Wood Declaration. Thus, Kong cannot render claim 50 as obvious. Therefore, applicants request that this rejection under 35 U.S.C. § 103 (a) be reconsidered and withdrawn.

CONCLUSIONS

Applicants respectfully submit that the pending claims are now in condition for allowance. The Examiner is hereby invited to contact the undersigned for any remaining issues.

Respectfully submitted,

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Epitope and isotype specificities of antibodies to β-amyloid peptide for protection against Alzheimer's disease-like neuropathology

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Transgenic PDAPP mice, which express a disease-linked isoform of the human amyloid precursor protein, exhibit CNS pathology that is similar to Alzheimer's disease. In an age-dependent fashion, the mice develop plaques containing β-amyloid peptide (Aβ) and exhibit neuronal dystrophy and synaptic loss. It has been shown in previous studies that pathology can be prevented and even reversed by immunization of the mice with the Aβ peptide. Similar protection could be achieved by passive administration of some but not all monoclonal antibodies against Aβ. In the current studies we sought to define the optimal antibody response for reducing neuropathology. Immune sera with reactivity against different Aβ epitopes and monoclonal antibodies with different isotypes were examined for efficacy both *ex vivo* and *in vivo*. The studies showed that: (*i*) of the purified or elicited antibodies tested, only antibodies against the N-terminal regions of Aβ were able to invoke plaque clearance; (*ii*) plaque binding correlated with a clearance response and neuronal protection, whereas the ability of antibodies to capture soluble Aβ was not necessarily correlated with efficacy; (*iii*) the isotype of the antibody dramatically influenced the degree of plaque clearance and neuronal protection; (*iv*) high affinity of the antibody for Fc receptors on microglial cells seemed more important than high affinity for Aβ itself; and (*v*) complement activation was not required for plaque clearance. These results indicate that antibody Fc-mediated plaque clearance is a highly efficient and effective process for protection against neuropathology in an animal model of Alzheimer's disease.

Immunization of the transgenic PDAPP mice with β-amyloid peptide (Aβ)-derived immunogens results in an antibody response that facilitates the clearance of plaques within the central nervous system (CNS) (1–4). Although a number of mechanisms are likely to operate in this clearance response (5, 6), our previous findings strongly indicate that antibody-mediated, Fc-dependent phagocytosis by microglial cells and/or macrophages is important to the process (7). Importantly, a T cell response was not required for amyloid plaque clearance. When peripherally administered, antibodies against Aβ entered the CNS of PDAPP transgenic mice, decorated amyloid plaques, and induced plaque clearance. Comparing different antibodies in an *ex vivo* assay with sections of PDAPP or Alzheimer's disease (AD) brain, there was a strong correlation between those that produced *ex vivo* efficacy and those that were efficacious *in vivo*. Fc receptors on microglial cells were found to be key for the clearance response in this assay. However, it has been reported that antibody efficacy can also be obtained *in vivo* by mechanisms that are independent of Fc interactions (8). Studies have indicated that an antibody directed against the midportion of Aβ, which cannot recognize amyloid plaques, appears to bind to soluble Aβ and reduce plaque deposition (6). In addition, it has been reported recently that short-term treatment with this antibody improved performance in an object-recognition task without affecting amyloid burden (9).

To understand the parameters of an antibody response that are required for neuronal protection, several questions should be considered. Is neuronal protection associated with plaque clearance, or is it necessary for antibodies to capture soluble aggregates of Aβ to protect neurons against the directly toxic effects of the peptide? Does a clearance response depend on Fc receptor-mediated phagocytosis of Aβ after antibody binding or on complement receptor-mediated phagocytosis after antibody binding and complement activation? Alternatively, is a clearance response independent of antibody Fc receptor function?

In the current study we approached these questions by examining the influence of different antibody epitopes and isotypes on plaque clearance and neuronal protection. The studies took advantage of the fact that some epitopes of Aβ are preferentially available for antibody binding within plaques, whereas others are only available for antibody capture of the soluble peptide. In addition, the isotype of an antibody is important for either Fc- or complement-mediated phagocytosis of Aβ by microglial cells, because antibody isotype defines its affinity for Fc receptors as well as its ability to activate complement. If plaque clearance and/or neuronal protection do not depend on Fc-mediated processes, then the isotype of an antibody against Aβ should have little impact on efficacy. These studies provide insight for the design of antibodies with therapeutic potential.

Materials and Methods

Aβ Fragments. Peptides corresponding to Aβ1–5, Aβ3–9, Aβ5–11, and Aβ15–24 and the reverse sequence Aβ5–1 were synthesized contiguous to a 17-aa T cell epitope derived from ovalbumin (amino acids 323–339, ISQAVHAAHAEINEAGR) on a branched peptide framework (triple-lysine core with four peptide arms) to produce a multiantigen peptide as described (10). Polyclonal antibodies against Aβ1–42 (pAb 1–42) were raised and the Ig fraction was isolated as described (7). pAb-EL16, pAb-EL17, and pAb-EL20 were obtained from the sera of PDAPP mice immunized with peptides corresponding to Aβ1–7, Aβ15–24, and Aβ3–9, respectively, which had been synthesized on a branched framework as described above. pAb-EL26 was obtained from the sera of mice immunized with Aβ(7–1)-42. The peptides were synthesized by AnaSpec (San Jose, CA).

Monoclonal Antibodies (mAbs). The production of mAbs 10D5 and 6C6, which were raised against synthetic Aβ1–28 coupled to a carrier protein, has been described (11). mAbs 12B4, 2C1, 12A11, and 3A3 were raised against synthetic Aβ1–42 by using similar methodology except that hybridoma supernatants were screened by an RIA. All antibodies were purified by HPLC and were free of endotoxin (<1 endotoxin unit/mg protein) as

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Abbreviations: AD, Alzheimer's disease; Aβ, β-amyloid peptide; pAb, polyclonal Ab.

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NEUROSCIENCE

determined by the *Limulus* amoebocyte gel-clot assay (Associates of Cape Cod). The mAbs 3D6 and 21F12 were obtained as described (7), and 22D12 and 266 were raised against synthetic Aβ13–28 (12).

Epitope Mapping. Epitope mapping of the mAbs and pAbs was performed by using an ELISA that measured antibody binding to overlapping peptides (10 amino acid peptides offset by 1 residue) covering the entire Aβ1–42 sequence. The first 32 peptides were biotinylated at the C terminus, and the last 10 peptides were biotinylated at the N terminus. The biotinylated peptides were synthesized by Mimotopes (Clayton, Victoria, Australia) and captured on streptavidin-coated wells of a 96-well plate (Pierce).

Passive and Active Immunization Procedures. mAbs in PBS were given via passive administration (i.p. injection) at a dose of 10 mg/kg weekly for 6 months. For active immunization, 100 μg of Aβ fragment was administered by i.p. injection in complete Freund's adjuvant followed by boosts with 100 μg of peptide in incomplete Freund's adjuvant at 2 and 4 weeks, and monthly thereafter.

Antibody Binding to Aggregated and Soluble Aβ1–42. Serum titers (determined by serial dilution) and mAbs binding to aggregated synthetic Aβ1–42 were performed by ELISA as described (1). Soluble Aβ1–42 refers to the synthetic Aβ1–42 peptide sonicated in dimethyl sulfoxide. Serial dilutions of sera or mAb at 20 μg/ml were incubated with 50,000 cpm [^{125}I]Aβ1–42 (≈190 μCi/μmol; labeling with Iodogen reagent, Pierce) overnight at room temperature. Fifty microliters of a slurry containing 75 mg/ml protein A Sepharose (Amersham Pharmacia) and 200 μg of rabbit anti-mouse IgG (H+L) (Jackson ImmunoResearch) was incubated with the diluted antibodies for 1 h at room temperature, washed twice, and counted on a Wallac gamma counter (Perkin–Elmer). All steps were performed in RIA buffer consisting of 10 mM Tris, 0.5 M NaCl, 1 mg/ml gelatin, and 0.5% Nonidet P-40, pH 8.0.

***Ex Vivo* Assay.** Cryostat sections (10 μm in thickness) of PDAPP mouse brain were thaw-mounted onto round polylysine-coated coverslips and placed in the wells of 24-well tissue-culture plates. Microglial cells and antibodies were added to the wells and cultured for 24 h as described (7). After incubation, cultures were extracted with an 8 M urea buffer and frozen quickly. Total Aβ level in the cultures was determined by ELISA as described (13).

Statistical analyses were performed by using PRISM 3.0 software (GraphPad, San Diego).

Results and Discussion

Aβ Epitope: Epitopes Within the N Terminus of Aβ Are Important for Plaque Clearance and Reduction of Neuritic Pathology. We have shown previously that not all antibodies against Aβ can trigger plaque clearance *in vivo*. Efficacy can be predicted by the ability of antibodies to both bind plaques within unfixed sections of PDAPP or AD brains and trigger plaque clearance in an *ex vivo* assay (7). In the current study, a number of mAbs and pAbs directed against different epitopes of Aβ were examined for plaque reactivity and *ex vivo* efficacy. Only antibodies against epitopes within the N-terminal 11 aa of Aβ were found to be active in either regard (Table 1). These findings also illustrate that, of the five antibodies previously examined *in vivo*, those against N-terminal epitopes were effective in reducing plaque burden (pAb1–42, 3D6, and 10D5), whereas those against C-terminal epitopes were inactive (16C11 and 21F12) (7).

To extend these findings and further characterize epitopes within the N terminus of Aβ, a series of peptides were compared for their ability to trigger an efficacious antibody response *in vivo*. Twelve- to 13-month-old PDAPP mice were immunized with one of three N-terminal peptide fragments (Aβ1–5, Aβ3–9, or Aβ5–11) or a fragment derived from an internal region of the peptide (Aβ15–24) (Fig. 1*a*). The internal peptide Aβ15–24 encompasses the epitope of antibody 266, which exhibits high affinity for soluble Aβ (12), but as shown above it does not recognize plaques in sections of unfixed AD or PDAPP tissue (Table 1). Thus, it was of interest to determine whether a polyclonal response directed against this peptide could produce antibodies capable of plaque recognition or whether reactivity with soluble Aβ alone was sufficient to provide efficacy. In these studies, a peptide with reverse sequence (Aβ5–1) served as a negative control. The peptides were synthesized contiguous to a 17-aa T cell epitope derived from ovalbumin and presented in an identical multivalent configuration (see *Materials and Methods*). All the peptides (except Aβ5–1 reverse mer) produced sera that recognized aggregated synthetic Aβ1–42 by ELISA (Fig. 1*b*). In contrast, only sera against the N-terminal peptides were able to recognize Aβ within plaques; antisera against Aβ15–24 did not bind plaques despite strong reactivity with the synthetic aggregated peptide (Fig. 1*c*). There also were differences between the serum groups in their ability to capture soluble Aβ (Fig. 2*a*). Less than 30% of the sera from mice immunized with Aβ1–5 or Aβ3–9 captured the soluble peptide (27% and 5%, respectively). In contrast, sera from approximately half of the animals immunized with Aβ5–11 and all of those immunized with Aβ15–24 captured soluble Aβ1–42.

Table 1. Antibodies directed against epitopes within the N-terminal 11 aa of Aβ1–42 bind amyloid plaques and trigger phagocytosis in an *ex vivo* assay

Antibody	Epitope	Binds plaques (PDAPP)	Triggers *ex vivo* phagocytosis
3D6	1–5	++	++
pAb-EL16	1–7	++	++
pAb1–42	1–11	++	++
10D5	3–7	++	++
pAb-EL21	5–11	+	+
pAb-EL26	11–26	–	–
22D12	18–21	–	–
266	16–24	–	–
pAb-EL17	15–24	–	–
16C11	33–42	–	–
21F12	34–42	–	–

Both measures were scored on three-step visual rating system based on fluorescence intensity for the plaques and by degree of Aβ uptake as described (7).

Because the degree of Aβ deposition can vary greatly as PDAPP mice age, the *in vivo* study was designed with at least 30 animals per group. Efficacy data are shown for individual mice and expressed as the percentage of either amyloid burden or neuritic dystrophy relative to the mean of the control (set at 100%). Immunization with each of the three N-terminal peptides significantly reduced amyloid burden (46–61%, $P < 0.002$) (Fig. 2*b*). Furthermore, Aβ3–9 and Aβ5–11 significantly reduced neuritic pathology (34% and 41%, respectively; $P < 0.05$) (Fig. 2*c*). In contrast, immunization with Aβ15–24 provided no protection against either amyloid burden or neuritic pathology. These results further support the association between plaque binding and antibody efficacy. They also indicate that capture of soluble Aβ is not required for reduction of neuritic pathology, because the antibody response against Aβ3–9 provided strong plaque reactivity and the highest level of protection against neuronal dystrophy yet exhibited the weakest capacity for recognition of soluble peptide. These results, however, do not eliminate the possibility that antibodies specific for Aβ capture could provide efficacy at higher titers or over longer periods of

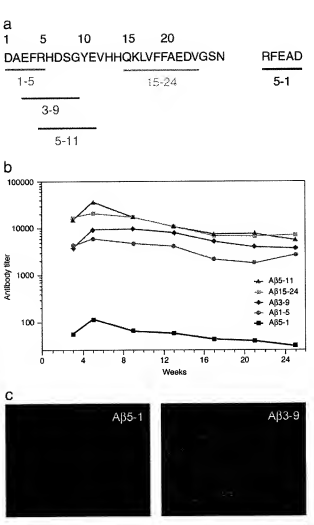

Fig. 1. Antibodies produced by immunization with N-terminal fragments of Aβ bind to amyloid plaques. (a) Peptides encompassing various domains of Aβ1–42 (synthesized contiguous to T cell epitope derived from ovalbumin) were used to immunize PDAPP mice. A reverse mer, Aβ5–1, was used as a negative control. (*b*) ELISA titers against aggregated Aβ1–42 were significantly higher over the length of the study in the Aβ5–11 and Aβ15–24 groups than in the Aβ1–5 group (t:14,457, $P < 0.01$, and t:12,257, $P < 0.05$ vs. t:3,647, respectively; ANOVA followed by post hoc Tukey's test). (*c*) Unfixed cryostat sections from untreated PDAPP mouse brain were exposed to the sera of mice immunized with Aβ5–1, Aβ3–9, Aβ5–11, or Aβ15–24 (titers normalized to 1:1,000 for staining). Antibodies to Aβ15–24 did not bind to amyloid plaques. (Scale bar, 500 μm.)

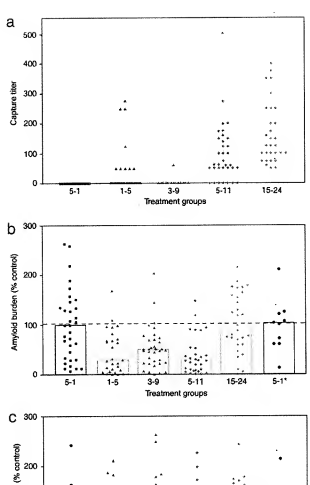

Fig. 2. Capture of soluble Aβ1–42 by antibodies is not associated with reduced amyloid burden or neuritic pathology. (a) Sera from mice immunized with fragments of Aβ were examined for their ability to capture radiolabeled soluble Aβ1–42 in an RIA. Sera from all animals immunized with Aβ15–24 were able to capture soluble Aβ1–42 (one serum sample had a titer higher than 1:1,350, and a precise titer was not determined) compared with 27% of those in the Aβ1–5 group and 3% of the Aβ3–9 group. Amyloid burden (*b*) and neuritic pathology (*c*) were evaluated with image analysis by a blinded microscopist. Values are expressed as a percentage of the mean of the Aβ5–1 group (negative control reverse mer peptide). The Aβ5–11 group was evaluated at a separate sitting from the other groups but in conjunction with the same negative control group as an internal reference (second Aβ5–1* set, on the right). Amyloid burden was reduced significantly in the Aβ1–5, Aβ3–9, and Aβ5–11 groups ($P < 0.001$). The bars represent median values, and the dashed horizontal line indicates the control level. Neuritic burden was reduced significantly in the Aβ3–9 and Aβ5–11 groups ($P < 0.05$). Neither endpoint was altered significantly by immunization with the Aβ15–24 group. Statistical analysis was performed with square-root transformation (to normalize nonparametric distributions) and analyzed with ANOVA. A Dunnett's test then was used to compare the multiple groups Aβ1–5, Aβ3–9, and Aβ15–24 with their Aβ5–1 control and Mann–Whitney for the Aβ5–11 group with its corresponding Aβ5–1* control.

time, as has been reported by DeMattos *et al.* (6) using the high-affinity capture antibody 266.

Antibody Isotype: IgG2a Antibodies Against Aβ Are More Efficient than IgG1 or IgG2b Antibodies in Reducing Neuropathology. Murine phagocytotic effector cells such as microglia within the CNS express three different classes of IgG-specific Fc receptors (FcγR receptors): a high-affinity receptor, FcγRI, and two low-affinity receptors, FcγRII and FcγRIII (14). FcγRII is a single-chain re-

Table 2. mAbs against Aβ3–7 have different avidity for aggregated and soluble synthetic Aβ1–42

Antibody	Epitope	Isotype	ED_{50} on aggregated Aβ1–42, pM	% Capture of soluble Aβ1–42
6C6	Aβ3–7	IgG1	40	1
10D5	Aβ3–7	IgG1	53	1
2C1	Aβ3–7	IgG2a	333	1
12B4	Aβ3–7	IgG2a	667	8
3A3	Aβ3–7	IgG2b	287	1
12A11	Aβ3–7	IgG2b	233	30

As a comparison, the antibody 266 at 10 μg/ml would capture 70% of Aβ1–42.

ceptor with two major isoforms that apparently lack phagocytic capacity (15). FcγRI and FcγRIII are heterooligomeric complexes in which the specific ligand-binding α chains are associated with a common γ chain. The precise contribution of FcγRI and FcγRIII to the phagocytosis of opsonized particles has not been defined; however, it has been shown that both receptors, and in particular FcγRI, exhibit a higher affinity for murine IgG2a than for IgG1 or IgG2b (16). Furthermore, IgG2a has proven to be more effective in a number of *in vivo* clearance responses than the other antibody isotypes (17–20). Thus, if Fc-mediated phagocytosis of Aβ peptide is an important mechanism for antibody-mediated plaque clearance, then IgG2a antibodies would be expected to reduce plaque burden more efficiently than the other antibody isotypes.

To address this issue, experiments were conducted with six mAbs: two of each IgG isotype and all directed against the same epitope of Aβ (Aβ3–7). The epitope was defined further by amino acid substitution analysis; each antibody required the same three residues within Aβ3–7 for binding, and each could tolerate substitution within these residues by similar amino acids (data not shown). All the antibodies exhibited high avidity for aggregated Aβ1–42 (<1 nM); however, the IgG1 antibodies showed ≈10-fold greater binding avidity than the IgG2a antibodies (≈50 vs. 500 pM) (Table 2). In contrast, only two of the antibodies could appreciably capture soluble Aβ1–42 at antibody concentrations of 20 μg/ml: one of the IgG2b antibodies (12A11) and, to a lesser extent, one of the IgG2a antibodies (12B4). As a measure of their ability to trigger Fc-mediated plaque clearance, the six antibodies were compared in the *ex vivo* assay with primary mouse microglial cells and sections of brain tissue from PDAPP mice. Irrelevant IgG1, IgG2a, and IgG2b antibodies, having no reactivity toward Aβ or other components of the assay, were used as isotype-matched negative controls. To quantify the degree of plaque clearance/Aβ degradation that occurred by the end of the assay, Aβ was extracted from the cultures of microglia and brain sections ($n = 3$) with 8 M urea for analysis by ELISA (*see Materials and Methods*). As shown in Fig. 3, the two IgG2a antibodies against Aβ reduced peptide levels in the cultures more efficiently (73% and 69%, $P < 0.001$) than the IgG1 (28% and 35%, not significant) or IgG2b (48% and 59%, $P < 0.05$ and 0.001, respectively) antibodies. Because previous studies showed that *ex vivo* plaque clearance depends on Fc-receptor activity (7) and can occur in the presence of heat-inactivated serum, it is unlikely that complement played a significant role in mediating the effects observed in the *ex vivo* assay.

The antibodies then were investigated for *in vivo* efficacy. Antibody (10 mg/kg) or PBS control was administered by weekly i.p. injection for 6 months as described (7). Antibodies within all groups maintained similar serum titers against aggregated Aβ1–42 (1:3,500) with the exception of the IgG1 antibody 10D5,

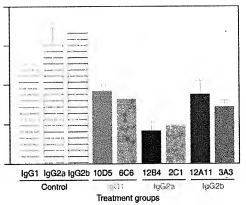

Fig. 3. Comparing antibodies against Aβ3–7 in the *ex vivo* assay, IgG2a antibodies clear β amyloid plaque more efficiently that either the IgG1 or IgG2b isotypes. Murine primary microglial cells were cultured with unfixed cryostat sections of PDAPP mouse brain in the presence of antibodies of different isotypes directed against Aβ3–7. Irrelevant IgG1, IgG2a, and IgG2b antibodies were used as the respective isotype-matched negative controls. After 24 h of incubation, the total level of Aβ remaining in the cultures was measured by ELISA. The two anti-Aβ IgG2a antibodies reduced Aβ levels in the cultures (69% for 2C1 and 73% for 12B4; $P < 0.001$) more efficiently than the IgG2b isotype antibodies (48% for 12A11, $P < 0.05$, and 59% for 3A3, $P < 0.001$). The anti-Aβ IgG1 antibodies did not significantly reduce Aβ levels. Data were analyzed with ANOVA followed by a post hoc Dunnett's test.

which displayed 3- to 4-fold higher titers (≈1:13,000). At the end of the study, total levels of cortical Aβ were determined by ELISA. Although each of the antibodies significantly reduced total Aβ levels compared with the PBS control ($P < 0.001$) (Fig. 4*a*), there was a trend toward greater levels of reduction by the two IgG2a antibodies (61% and 69% reduction) than by the IgG1 (38% and 52%) or the IgG2b (44% and 31%) antibodies. These results suggest that the higher affinity of IgG2a for Fc receptors is an important parameter for clearance. Furthermore, because murine IgG1 antibodies cannot fix complement but provided levels of Aβ clearance comparable to the complement-fixing IgG2b antibodies, complement does not seem to play a critical role during the clearance process *in vivo*.

The level of neuritic dystrophy then was examined in sections of brain tissue from the mice to determine the association between plaque clearance and neuronal protection. Again, data are shown for individual animals and expressed as the percentage of neuritic dystrophy relative to the mean of the control (set at 100%). Although all the antibodies triggered plaque clearance, only the IgG2a antibodies provided significant reduction in neuritic dystrophy (12B4, $P < 0.05$, and 2C1, $P < 0.001$) (Fig. 4 *b* and *c*). Interestingly, the antibody 10D5 (IgG1) was less effective than either of the IgG2a antibodies even though it exhibited higher avidity for aggregated Aβ1–42 (Table 2) as well as amyloid plaques (data not shown) and maintained significantly higher serum titers than the other antibodies. Thus, antibody isotype and affinity for Fc receptors seem to be important attributes for both clearance of Aβ and protection against neuritic dystrophy and may be more important than the relative avidity of antibodies for Aβ1–42 (Table 2). Also, these studies confirmed the observation obtained in the peptide immunization study described above that antibodies do not need to strongly capture soluble Aβ1–42 to provide protection against neuritic dystrophy. The antibody 12A11 (IgG2b) captured soluble monomeric Aβ1–42 more efficiently than either of the IgG2a antibodies (Table 1) but was not as effective. Also, both IgG2a antibodies provided similar protection even though only one could capture the soluble peptide detectably. The results of the current study are not necessarily inconsistent with other

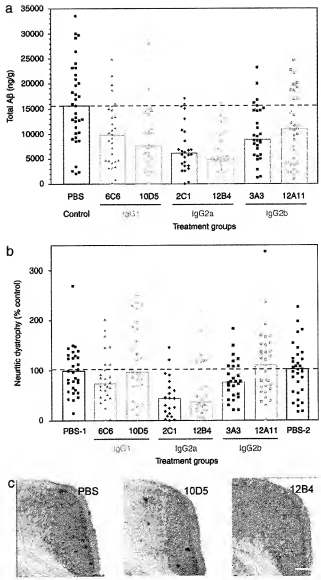

Fig. 4. Anti-Aβ IgG2a antibodies reduced AD-like neuropathology more efficiently than other isotypes *in vivo*. PDAPP mice received weekly i.p. injections of antibodies starting at 12 months of age for 6 months. (*a*) Total Aβ levels shown for individual mice sorted by treatment group (n = 30). The bars represent median values, and the dashed horizontal line indicates the control level. Although Aβ levels were reduced significantly in all antibody groups ($P < 0.001$ vs. PBS; ANOVA followed by post hoc Dunnett's test), the IgG2a groups exhibited the highest degree of clearance. (*b*) The percentage of frontal cortex occupied by neuritic dystrophy was determined by image analysis. The different groups within this experiment were analyzed in two sets by using the same PBS group as an internal standard (PBS-1 and PBS-2). PBS-1 was the control for the 6C6, 10D5, 12B4, and 12A11 groups, and PBS-2 was the control for the 2C1 and 3A3 groups. To compare the groups, values for individual animals are expressed as a percentage of the mean of their respective PBS control group (set at 100%). The bars represent median values, and the dashed horizontal line indicates the control level. Neuritic dystrophy was reduced significantly only by the IgG2a isotype antibodies (12B4, $P < 0.05$, and 2C1, $P < 0.001$; ANOVA followed by post hoc Dunnett's test). (*c*) Dystrophic neurites were labeled with the amyloid precursor protein-specific antibody 8E5, and were found in association with plaques. Relative to PBS control, the neuritic pathology was reduced significantly in animals treated with 12B4 but not 10D5. (Scale bar, 250 μm.)

investigations and hypotheses surrounding anti-Aβ-based immunotherapies. Duration of treatment, route of administration, and specific antibody properties all likely have important effects on the observed outcomes. For example, Solomon *et al.* (5) suggested that anti-Aβ antibodies may directly inhibit or reverse amyloid fibril formation. Bacskai *et al.* (8) provided support for the possible direct dissolution mode of action by showing removal of plaque *in vivo* by F(ab')$_2$ fragments of an anti-Aβ antibody after direct application to the brain. It should be noted, however, that *in vitro* dissolution was reported to be restricted to antibodies against Aβ3–6 (21), whereas the antibody used in the *in vivo* study was against Aβ1–5. In addition, we show that antibodies against fragments Aβ1–5, Aβ3–9, and Aβ5–11 are all capable of reducing plaque burden. Thus the mechanism of plaque reduction does not seem to have the same restricted epitope as reported for *in vitro* fibril dissolution. Although the direct application of high-dose antibody to brain was capable of clearing plaque without Fc-mediated phagocytosis, the present data demonstrate that the efficiency in reducing plaque burden and neuritic dystrophy is best when the antibody isotype maximally supports phagocytosis and that efficacy can be achieved by antibodies of several epitope specificities that all are capable of binding plaque *in vivo* or *ex vivo*.

Another reported mechanism of efficacy is through capture of soluble Aβ (6). The use of antibody 266, a high-affinity capture antibody, at concentrations sufficient to produce detectable cerebrospinal fluid levels reduced plaque burden after chronic treatment. It is likely that the capture titer achieved by immunization with the Aβ15–24 fragment, although still substantially greater than the other immunogens tested in this study, is less than that achieved by 266 dosing, and this may impact the outcome. Thus it may be possible to achieve the same endpoint through multiple mechanisms including a chronic capture of Aβ species, dissolution of plaques, or phagocytosis of existing aggregates.

In summary, although antibodies against Aβ may exhibit efficacy in a number of ways, protection against AD-like neuropathology can be obtained by antibodies that bind to plaques and trigger Fc-mediated clearance. IgG2a antibodies, which exhibit higher affinity than other isotypes for phagocytic Fc receptors (in particular FcγRI), provided the highest level of plaque clearance and were the only anti-Aβ antibodies to provide neuronal protection under the conditions tested. Plaque clearance seemed independent of complement activation, because IgG1 antibodies, which cannot fix complement, were as effective as the complement-fixing IgG2b antibodies. These results are consistent with the role of high-affinity Fc receptors in other clearance systems, where they have been shown to be particularly effective for inducing clearance in conditions with low antibody concentrations (as would be anticipated in the CNS). The density of target-bound IgG that is required for complement activation has been reported to be higher than that required for Fc receptor-mediated phagocytosis (17). Accordingly, complement involvement in plaque clearance may be more pronounced at higher doses of antibody, where there would be an increased density of antibody bound to plaques. Interestingly, in contrast to other macrophage-activation paradigms, it has been shown that activation of phagocytic cells through Fc receptors results in production of the antiinflammatory cytokine IL-10 and inhibition of proinflammatory IL-12 (22). Thus, antibodies against Aβ may allow resolution of an otherwise chronic, unresolving inflammatory response associated with plaques in AD by both clearing Aβ and altering the inflammatory environment.

In addition, the current studies demonstrate that antibody epitopes within the N terminus of Aβ are important for plaque clearance and neuronal protection via an Fc-mediated mechanism. Passive administration of mAbs against defined Aβ epitopes reduced plaque burden and neuritic pathology to the same degree as active immunization. Although the 5- to 7-aa residue epitopes used for immunization in our studies are

themselves too short to elicit T cell help for antibody production, we showed that these epitopes can be synthesized in conjunction with an exogenous T cell epitope to produce an efficacious antibody response after administration. Such an approach will avoid generation of T cell immunity against Aβ as a self-antigen and may preclude the potential issues with encephalitis that were observed recently with a subset of patients in the clinic after immunization with whole Aβ1–42 (23, 24). Likewise, T cell immunity will not be elicited with passive administration of antibodies against Aβ. Thus, both immunoconjugates containing defined epitopes of Aβ and mAbs against appropriate Aβ epitopes offer excellent alternatives to whole-peptide immunization for the treatment of AD.

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